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Endocytosis of the AT_{1A} angiotensin receptor is independent of ubiquitylation of its cytoplasmic serine/threonine-rich region

Balázs Mihalik[a], Zsuzsanna Gáborik[a], Péter Várnai[a], Adrian J.L. Clark[b], Kevin J. Catt[c], László Hunyady[a,*]

[a] *Department of Physiology, Faculty of Medicine, Semmelweis University, P.O. Box 259, H-1444 Budapest, Hungary*
[b] *Department of Endocrinology, St. Bartholomew's and the Royal London School of Medicine and Dentistry, West Smithfield, London, UK*
[c] *Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-4510, USA*

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Abstract

Agonist-induced internalisation of the rat type 1A (AT_{1A}) angiotensin II receptor is associated with phosphorylation of a serine/threonine-rich region in its cytoplasmic tail. In yeast, hyperphosphorylation of the α-factor pheromone receptor regulates endocytosis of the receptor by facilitating the monoubiquitylation of its cytoplasmic tail on lysine residues. The role of receptor ubiquitylation in AT_{1A} receptor internalisation was evaluated by deletion or replacement of lysine residues in its agonist-sensitive serine/threonine-rich region. Expression of such receptor mutants in CHO cells showed that these modifications had no detectable effect on the angiotensin II-induced endocytosis of the AT_{1A} receptor. Furthermore, fusion of ubiquitin in-frame to an internalisation-deficient AT_{1A} receptor mutant with a truncated carboxyl-terminal tail did not restore the endocytosis of the resulting chimeric receptor. No impairment of receptor internalisation was observed after substitution of all lysine residues in the serine/threonine-rich region at saturating angiotensin II concentrations, where endocytosis occurs by a β-arrestin and dynamin independent mechanism. Taken together, these data demonstrate that ubiquitylation of the cytoplasmic serine/threonine-rich region of the AT_{1A} receptor on lysine residues is not required for its agonist-induced internalisation, and suggest that endocytosis of mammalian G protein-coupled receptors (GPCRs) occurs by a different mechanism than that of yeast GPCRs.

Keywords: AT_{1A} angiotensin receptor; Carboxyl-terminal tail; Internalisation; Receptor regulation; Ubiquitin

1. Introduction

The type 1 (AT_1) angiotensin receptor is a seven transmembrane receptor that mediates the major physiological effects of the octapeptide hormone, angiotensin II (Ang II). The rodent AT_1 receptor has two closely related subtypes, the AT_{1A} and AT_{1B} receptors, with similar signalling and ligand binding properties and distinct tissue distributions (Hunyady, Balla, & Catt, 1996; De Gasparo, Catt, Inagami, Wright, & Unger, 2000). Ang II binding to AT_1 receptors stimulates phospholipase C activation and Ca^{2+}/protein kinase C signalling via $G_{q/11}$ proteins, and other signalling events through small GTP-binding proteins (Hunyady et al., 1996; De Gasparo et al., 2000). Like many other G protein-coupled receptors (GPCRs),

* Corresponding author. Tel.: +36-1-266-2755x4041; fax: +36-1-266-6504.
E-mail address: hunyady@puskin.sote.hu (L. Hunyady).

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the signalling functions of the AT_{1A} receptor are regulated by phosphorylation, desensitisation, and internalisation of the hormone–receptor complex (Thomas, 1999; Hunyady, Catt, Clark, & Gaborik, 2000; Conchon, Peltier, Corvol, & Clauser, 1998; Vinson et al., 1995). Agonist-induced activation of GPCRs causes phosphorylation of specific serine/threonine residues by GPCR kinases (GRKs), which facilitates the binding of arrestins and promotes desensitisation by uncoupling the receptor from its cognate G proteins (Krupnick & Benovic, 1998; Ferguson, 2001; Claing, Laporte, Caron, & Lefkowitz, 2002). In the case of the AT_{1A} receptor, binding of angiotensin II causes phosphorylation of a serine/threonine-rich domain in the carboxyl-terminal tail of the receptor (Smith et al., 1998; Thomas, Motel, Kule, Karoor, & Baker, 1998) and enhances its association with β-arrestins (Qian, Pipolo, & Thomas, 2001).

Another function of agonist-stimulated β-arrestin binding to mammalian GPCRs is the regulation of receptor internalisation. β-Arrestins can serve as adaptor molecules that link the activated receptor to the endocytotic machinery via their C-terminal clathrin and/or AP2 adaptor protein binding domains (Ferguson et al., 1996; Goodman et al., 1996; Laporte et al., 1999). This appears to be the predominant mechanism of AT_{1A} receptor internalisation that is operative at physiological Ang II concentration (Gáborik et al., 2001). In yeast cells, however, agonist-induced phosphorylation of pheromone receptors promotes monoubiquitylation of the receptor at lysine residues, and this covalent modification regulates receptor internalisation (Terrell, Shih, Dunn, & Hicke, 1998).

Ubiquitin is a 76-amino-acid polypeptide that is conjugated to target proteins by the formation of an isopeptide bond between its C-terminal glycine residue and the ε-amino groups of lysine residues in the substrate protein by the sequential actions of three enzymes (Hershko & Ciechanover, 1998). The ubiquitylation of cytosolic and nuclear proteins leads to their degradation in proteasomes (Bonifacino & Weissman, 1998). However, ubiquitylation of plasma membrane proteins has been shown to target these molecules to the endocytotic pathway in yeast and in mammalian cells (Bonifacino & Weissman, 1998; Hicke, 1999). In yeast strains with mutations in the ubiquitin conjugating system, the α-factor receptor does not undergo ubiquitylation in response to pheromone exposure and shows multiple defects in its internalisation process (Hicke & Riezman, 1996). Also, endocytosis of the α-factor receptor was abolished when all lysine residues in its cytoplasmic tail were mutated. Although many lysine residues in the cytoplasmic tail of the α-factor receptor can serve as ubiquitin conjugating sites, its internalisation is dependent on the ubiquitylation of a lysine residue in a well-defined SINNDAKSS endocytosis signal sequence (Hicke & Riezman, 1996). Binding of α-factor causes hyperphosphorylation of serine and threonine residues in the carboxyl-terminal cytoplasmic tail of its receptor, and ubiquitylation of the lysine residue in the SINNDAKSS sequence is regulated by phosphorylation of the adjacent serine residues (Hicke, Zanolari, & Riezman, 1998). Fusion of ubiquitin in-frame rescued the impaired internalisation of tail deletion mutant α-factor receptors, and stimulated the internalisation of a stable membrane protein (Terrell et al., 1998; Shih, Sloper–Mould, & Hicke, 2000). These studies led to the conclusion that monoubiquitylation of activated α-factor receptors can serve as an internalisation signal. A similar mechanism has been described for the receptor of the other yeast mating pheromone, the **a**-factor. Ubiquitylation and internalisation of the **a**-factor receptor is regulated by a large PEST-like sequence (Roth, Sullivan, & Davis, 1998). In yeast cells, the internalised pheromone receptors are transported to the lysosome-like vacuole and are inactivated by degradation. Several mammalian transmembrane proteins that are degraded in lysosomes also undergo ubiquitylation at the cell surface. These include the epidermal growth factor (EGF) receptor, the growth hormone receptor, and epithelial Na^+-channels (Bonifacino & Weissman, 1998; Hicke, 1999). Recent studies have suggested that interaction of the ubiquitin moiety in ubiquitylated membrane proteins with components of the endocytic machinery may contribute to the recruitment of endocytosed proteins into clathrin-coated pits (Polo et al., 2002; Soubeyran, Kowanetz, Szymkiewicz, Langdon, & Dikic, 2002). Although the above data demonstrate that ubiquitylation is required for the internalisation of membrane proteins, ubiquitylation of the $β_2$-adrenergic receptor and the CXCR4 chemokine receptor is not required for their internalisation (Shenoy, McDonald, Kohout, & Lefkowitz, 2001; Marchese & Benovic, 2001), and the potential role of this mechanism during endocytosis of other mammalian GPCRs has not been elucidated.

At physiological Ang II concentrations agonist-induced internalisation of the AT_{1A} receptor occurs via clathrin-coated pits by a dynamin- and β-arrestin-dependent mechanism (Qian, Pipolo, & Thomas, 2001; Gáborik et al., 2001; Anborgh, Seachrist, Dale, & Ferguson, 2000; Kohout, Lin, Perry, Conner, & Lefkowitz, 2001). However, this process appears to be dynamin- and β-arrestin independent at saturating Ang II concentrations (Gáborik et al., 2001; Zhang, Ferguson, Barak, Menard, & Caron, 1996). Mutational analysis of the AT_{1A} receptor has shown that a serine/threonine-rich region in its cytoplasmic tail is required for its agonist-induced endocytosis (Hunyady, Bor, Balla, & Catt, 1994b; Thomas, Baker, Motel, & Thekkumkara, 1995a,b), and recent studies have mapped the main agonist-induced phosphorylation sites to this region of the receptor (Smith et al., 1998; Thomas, Motel, Kule, Karoor, & Baker, 1998). This serine- and threonine-rich region (STKMSTLS) in the C-terminal tail of the AT_{1A} receptor also contains a lysine residue and resembles the SINNDAKSS sequence of the α-factor receptor (Fig. 1). This similarity between these sequences and the regulatory role of agonist-induced phosphorylation of this region in AT_1 receptor endocytosis raised the question whether ubiquitylation of this region has a similar role in receptor internalisation as that of the α-factor receptor. The present study was designed to investigate the role of ubiquitylation of these C-terminal lysine residues in the regulation of AT_{1A} receptor endocytosis.

2. Materials and methods

2.1. Materials

Cell culture media, transfection reagents, and fetal bovine serum were purchased from Invitrogen Inc. ^{125}I-angiotensin II was from Covance. Unless otherwise stated, reagents were obtained from Sigma or Fluka.

2.2. Construction of receptor mutants

Mutations were introduced into the sequence of the rat AT_{1A} receptor cDNA cloned into the pcDNA3.1/Amp (Invitrogen Inc.) eukaryotic expression vector using the QuikChange site directed mutagenesis kit (Stratagene). The 319STOP receptor mutant was created as described previously (Hunyady et al., 1994b). One or more lysine residues in the C-terminal tail of the wild-type and 338STOP mutant AT_{1A} receptor were substituted with alanine as detailed in Section 3. To facilitate the identification of the mutant plasmids by restriction enzyme analysis, the designed oligonucleotides contained silent mutations to add or remove restriction enzyme digestion sites. After an initial screening with restriction enzymes, all mutations were confirmed by sequencing using the ThermoSequenase sequencing kit (Amersham Biosciences).

The ubiquitin-tagged 319STOP AT_{1A} receptor mutant was generated by a recombinant polymerase chain reaction (PCR) technique. Full-length human ubiquitin cDNA amplified from a human EST clone (Research Genetics Inc.) was fused to the 3′-terminus of a fragment containing the AT_{1A} receptor cDNA from the EcoRI site to the Lys^{318} amino acid of the receptor, by PCR. This PCR product was ligated into a pcDNA3.1/AT_{1A} plasmid replacing the wild-type EcoRI/NotI fragment of the AT_{1A} receptor cDNA with the ubiquitin fusion fragment. Between the truncated receptor and the ubiquitin coding sequences there is a short glycine linker sequence as shown in Fig. 1. Mutations in the ubiquitin molecule to convert Lys^{48} to Arg and Gly^{76} to Ala were introduced with a QuikChange site-directed mutagenesis kit (Stratagene). The in-frame fusion and the mutations in the ubiquitin sequence were verified by dideoxy sequencing.

2.3. Cell culture and transfection

CHO K-1 cells were maintained in Ham's F12 medium containing 10% fetal calf serum (FCS), 100 mg/ml streptomycin and 100 IU/ml penicillin (Invitrogen Inc.). Transient transfections were performed in 24-well plates using Lipofectamine (Invitrogen Inc.) as described previously (Gáborik et al., 2001; Hunyady, Bor, Baukal, Balla, & Catt, 1995).

2.4. Receptor internalisation

Agonist-induced internalisation of the AT_{1A} receptor was determined 48 h after transfection as described previously (Gáborik et al., 2001; Hunyady,

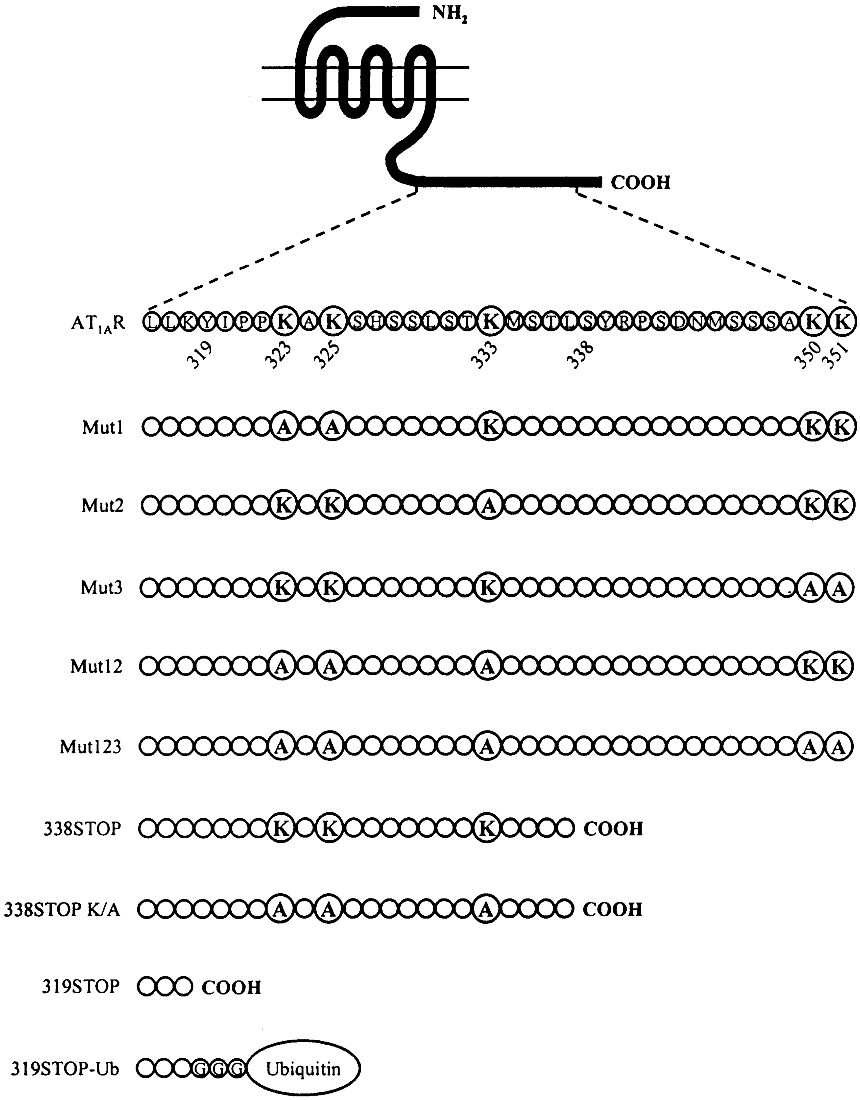

Fig. 1. Cytoplasmic tail mutants of the rat AT_{1A} receptor. In the schematic representation of the wild-type AT_{1A} receptor, the analysed region within the carboxyl terminus of the receptor is shown by the one letter amino acid codes. The Lys^{323}, Lys^{325}, Lys^{333}, Lys^{350} and Lys^{351} residues indicated in bold were mutated to alanine. The 338STOP and 319STOP mutants were generated by inserting STOP codons after Leu^{337} and Lys^{318}. The 319STOP-Ub mutant was created by an in-frame fusion of human ubiquitin cDNA to the carboxyl terminus of the 319STOP AT_{1A} receptor mutant with a short linker sequence containing three glycine residues.

Baukal, Balla, & Catt, 1994a). Briefly, cells were incubated in the presence of ^{125}I-Ang II (30–50 pM or 100 and 30 nM unlabelled Ang II) for the indicated times in HEPES-buffered F12 at 37 °C. The cells were then washed twice with ice-cold PBS and the internalised and surface-bound ligands were separated by removing the extracellular ligand with an acidic solution. The internalised labelled ligand was removed from the plates by solubilisation of the cells in an SDS/NaOH solution. The acid-sensitive and acid-resistant cell-associated radioactivity was determined by γ-spectrometry. The internalised specific binding is shown as percent of the total specific binding at each time point.

3. Results

Mutant AT_{1A} receptors were created to investigate the role of ubiquitylation of C-terminal lysine residues in receptor endocytosis. Five lysine residues are present in the serine/threonine-rich region of the C-terminal tail of the receptor (Fig. 1), and five additional residues (Lys^{307}, Lys^{308}, Lys^{310}, Lys^{311} and Lys^{318}) are located in the C-terminal tail proximal to this region. A role of ubiquitylation of the latter residues in AT_{1A} receptor internalisation is unlikely, given the lack of adjacent serine and threonine residues. Furthermore, studies in yeast have shown that substitution of the C-terminal tail of the α-factor receptor at its proximal region with ubiquitin only supports receptor endocytosis with markedly impaired kinetics (Shih et al., 2000). Earlier work from our group indicated that membrane-proximal lysine residues in the C-terminal tail are important for normal expression of the receptor in COS-7 cells, but these amino acids do not play a major role in the signalling and internalisation of the receptor (Gáborik et al., 1998). Furthermore, truncation of the receptor carboxyl-terminal tail sequence at the Tyr^{319} residue, which is upstream of the serine/threonine-rich region (Fig. 1), substantially inhibits its agonist-induced internalisation (Hunyady et al., 1994b; Thomas, Thekkumkara, Motel, & Baker, 1995a). For this reason, the present study focused on the five lysine residues in the serine/threonine-rich distal region of the cytoplasmic tail.

These lysine residues are distributed in the C-terminal tail at three locations (Fig. 1). Two of them (Lys^{323} and Lys^{325}) are within the N-terminal portion of the serine/threonine-rich region, and two adjacent lysines (Lys^{350} and Lys^{351}) are located in the C-terminal part of this region. A single lysine residue (Lys^{333}) is located between these groups adjacent to the Ser^{335}-Thr^{336}-Leu^{337} motif, which is critical for AT_{1A} receptor internalisation (Hunyady et al., 1994b). Substitution of this residue with alanine was previously found to have no major effect on the initial rate of AT_{1A} receptor internalisation (Hunyady et al., 1994b). Double alanine substitutions of the two pairs of lysine residues (Mut1 and Mut3; AT_{1A} receptor mutants are defined in Fig. 1) were created by site-directed mutagenesis. These constructs and the Mut2 receptor were expressed in CHO cells, and the internalisation rate of labelled Ang II was analysed in these cells as described in Section 2. The internalisation of labelled Ang II in these mutants was identical with that of the wild-type AT_{1A} receptor (Fig. 2A).

In the yeast system, ubiquitylation occurs at several lysine residues and elimination of individual residues does not detectably alter the endocytosis of the α-factor receptor (Terrell et al., 1998). To exclude the possibility that the mutant receptor is ubiquitylated at another lysine residue of the serine/threonine-rich region, combined lysine to alanine mutant AT_{1A} receptors were created. Substitution of three lysine residues with alanine (Mut12) or all five lysine residues with alanine (Mut123) had no detectable effect on the endocytosis of the AT_{1A} receptor (Fig. 2B). These results suggest that clathrin-mediated internalisation of the AT_{1A} receptor does not require ubiquitylation of the lysine residues in the serine/threonine-rich region of the cytoplasmic tail.

Although there are eight lysine residues in the α-factor receptor tail, only two of these (Lys^{337} and Lys^{347}) serve as a strong ubiquitylation signal. A truncated α-factor receptor (345Stop) lacking two-thirds of its cytoplasmic tail was found to internalise with similar kinetics as the wild-type receptor. Substitution of Lys^{337} in the SINNDAKSS sequence of the truncated receptor to arginine completely abolished internalisation of the receptor–ligand complex, while replacement of another lysine residue (Lys^{304}) in the truncated receptor had no effect (Shih et al., 2000). In the case of the AT_{1A} receptor, we have previously shown that deletion of the distal portion of the C-terminal tail by substituting Ser^{338} with a triplet encoding a STOP codon has no major effect on the internalisation kinetics of the receptor (Hunyady et al., 1994b). Similar to the 345STOP mutant in yeast (Terrell et al., 1998), the 338STOP mutation of the AT_{1A} receptor is adjacent to the serine/threonine-rich region required for receptor internalisation, and eliminates potential distal ubiquitylation sites by removing Lys^{350} and Lys^{351}. To further investigate the possible role of receptor ubiquitylation in AT_{1A} receptor internalisation, alanine substitutions of the remaining lysine residues were performed in the deletion mutant receptor. As shown in Fig. 3, substitution

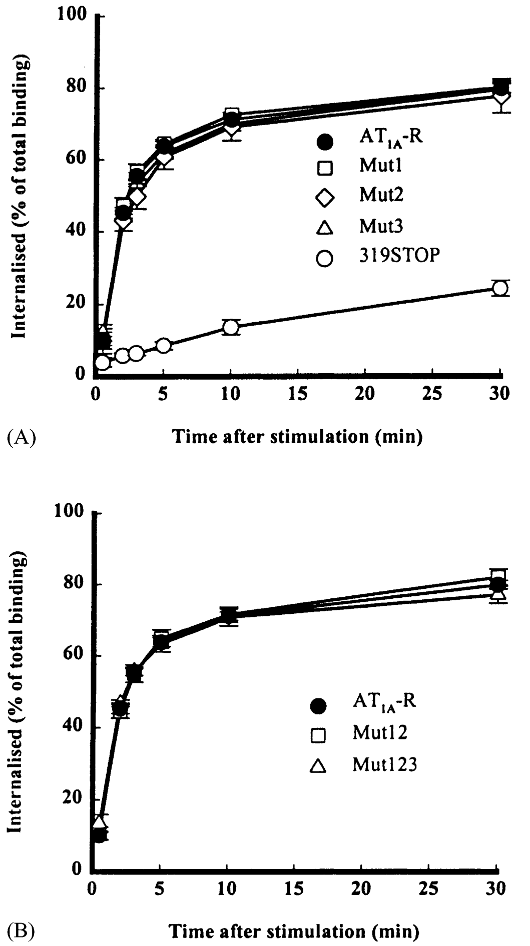

Fig. 2. Internalisation kinetics of the full-length and 319STOP tail deletion receptor mutants in CHO cells under physiological ligand concentration. (A) The Mut1 (□), Mut2 (◇), Mut3 (△), 319STOP (○) AT_{1A} receptor mutants and the wild-type (●) receptor were transiently expressed in CHO cells. Cells were incubated with ^{125}I-Ang II for the indicated periods at 37 °C after 48 h transfection and the internalised radioactivity was determined as described in Section 2. The internalised, labelled ligand was calculated as percent of the total specific binding. Data are shown as mean ± S.E. from three independent experiments each performed in duplicate. (B) Internalisation of transiently expressed AT_{1A} wild-type (●) and Mut12 (□), Mut123 (△) mutant receptors in CHO cells. Receptor internalisation was measured after addition of ^{125}I-Ang II at zero time at 37 °C and internalised binding was determined as described above. Values of total (extracellular plus internalised) specific binding after 30 min incubation at 37 °C for wild-type, Mut1, Mut2, Mut3, Mut12, Mut13, and 319STOP mutant AT_{1A} receptors were 13.2 ± 2.0, 8.1 ± 1.3, 15.5 ± 4.9, 10.9 ± 2.3, 8.3 ± 0.8, 11.8 ± 3.3, and 10.7 ± 3.3%, respectively, of the added tracer. Each point represents the mean ± S.E. of three independent experiments, each performed in duplicate.

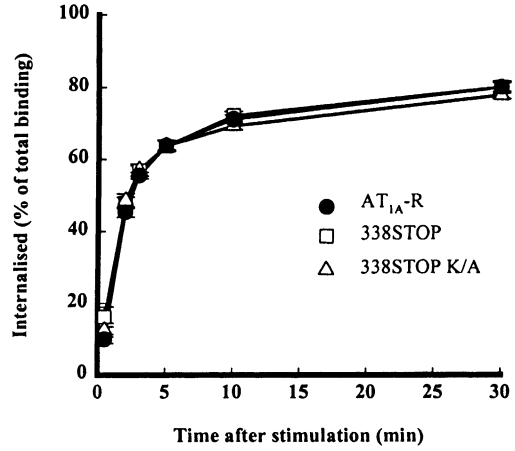

Fig. 3. Internalisation of the truncated tail mutant AT_{1A} receptor (338STOP) and the 338STOP K/A receptor mutant in CHO cells. Cells were transiently transfected with 338STOP K/A (△) and 338STOP (□) receptor mutants and wild-type (●) AT_{1A} receptor. Internalisation of ^{125}I-Ang II was determined as described in Section 2. Values of total (extracellular plus internalized) specific binding after 30 min incubation at 37 °C for wild-type, 338STOP and 338STOP K/A mutant AT_{1A} receptors were 13.2 ± 2.0, 13.9 ± 2.4 and 12.3 ± 4.1%, respectively, of the added tracer. Data represent the mean ± S.E. of three independent experiments, each performed in duplicate.

of Lys^{323}, Lys^{325} and Lys^{333} in the deletion mutant did not change the internalisation kinetics of labelled Ang II. Thus, similar to the previous set of experiments, removal of all five lysine residues in the serine/threonine-rich region of the cytoplasmic tail of the AT_{1A} receptor had no effect on its endocytosis.

To further evaluate the possible function of ubiquitylation of the receptor, we constructed a ubiquitin-tagged receptor mutant (319STOP-Ub) by fusing human ubiquitin to the carboxyl-terminus of the 319STOP receptor mutant. We have previously shown that the carboxyl-terminal region of the intracellular tail of the AT_{1A} receptor contains sequence determinants required for endocytosis of the receptor (Hunyady et al., 1994b). In the AT_{1A} receptor, truncation of the C-terminal tail by substituting Tyr^{319} with a STOP codon caused impaired internalisation of the receptor in COS-7 cells (Hunyady et al., 1994b; Thomas et al., 1995a). Similar to the yeast studies, we also introduced two mutations into the ubiquitin molecule. Substitution of arginine for Lys^{48} was performed to remove the main lysine residue required for further ubiquitin attachment, and to prevent the chimeric protein from polyubiquitylation. Also, ubiquitin's Gly^{76} residue was replaced by alanine to protect the fusion receptor from the action of de-ubiquitylating enzymes (Shih et al., 2000). Whereas the K48R mutation abolishes polyubiquitylation required for proteasomal degradation of the protein, the G76A mutation prevents removal of the ubiquitin moiety. To investigate the contribution of ubiquitin to AT_{1A} receptor endocytosis, we analysed the internalisation kinetics of this receptor chimera. We hypothesized that in the non-internalised 319STOP AT_{1A} receptor mutant, internalisation should be rescued by attachment of a ubiquitin molecule to its carboxyl-terminal end, as observed for the α-factor receptor (Shih et al., 2000), if ubiquitin is involved in the internalisation of the AT_{1A} receptor. However, the internalisation kinetics of the AT_{1A} receptor mutant-ubiquitin chimera did not differ from those of the 319STOP mutant (Fig. 4). This observation further indicated that attachment of ubiquitin to the receptor does not affect the kinetics of AT_{1A} receptor internalisation.

We have previously shown that the mechanism of AT_{1A} receptor differs according to the agonist concentrations (Gáborik et al., 2001). At physiological Ang II concentrations, β-arrestin- and dynamin-dependent endocytosis is the predominant mechanism of AT_{1A} receptor internalisation. However, at saturating hormone concentrations a β-arrestin- and dynamin-independent mechanism appears to operate (Gáborik et al., 2001). To test whether receptor ubiquitylation plays a role in the latter mechanism of AT_{1A} receptor internalisation, we analysed the endocytosis of wild-type and Mut123 mutant AT_{1A} receptors at saturating Ang II concentrations. In contrast to previous experiments, in which the concentration of radiolabelled agonist was low (0.03 nM), the labelled Ang II was increased to 0.1 nM and the total Ang II concentration was increased to 30 nM. Internalisation kinetics were analysed under these conditions in CHO cells transiently transfected with Mut123 receptor

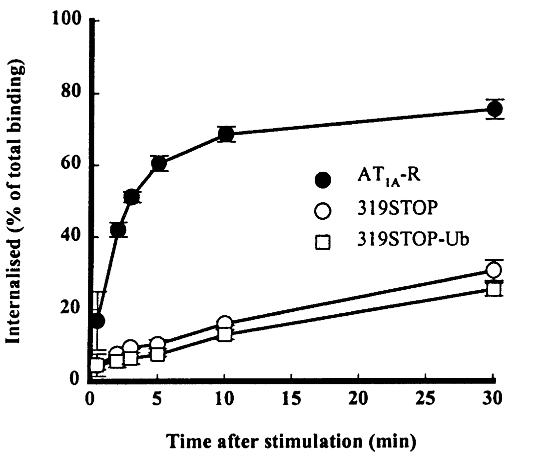

Fig. 4. Internalisation kinetics of wild-type AT_{1A} receptor (●), 319STOP (○) and 319STOP-Ub (□) receptor mutants in transiently transfected CHO cells. Cells were incubated with ^{125}I-Ang II for the indicated periods at 37 °C after 48 h transfection and the internalised radioactivity was determined as described in Section 2. The internalised, labelled ligand was calculated as a percent of total specific binding. Values of total (extracellular plus internalised) specific binding after 30 min incubation at 37 °C for wild-type, 319STOP and 319STOP-Ub mutant AT_{1A} receptors were 15.3 ± 2.9, 5.8 ± 1.3 and 7.0 ± 2.0%, respectively, of the added tracer. Data are shown as mean ± S.E. from three independent experiments, each performed in duplicate.

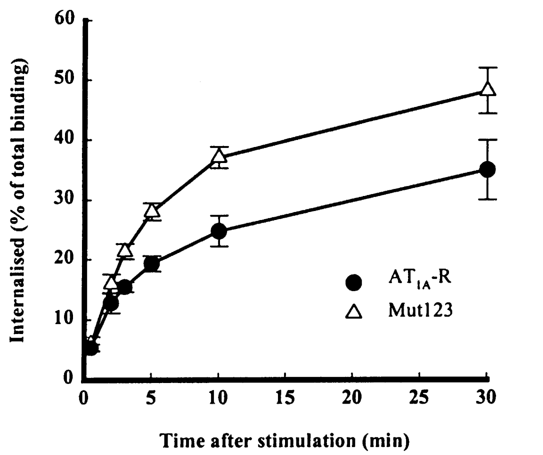

Fig. 5. Internalisation of the full-length Mut123 (△) mutant and the wild-type AT_{1A} receptor in the presence of 30 nM radiolabelled agonist. CHO cells were transiently transfected with the appropriate cDNA construct, 48 h after transfection cells were incubated with ^{125}I-Ang II added to radioinert Ang II to bring the final concentration to 30 nM. Internalisation of labelled ligand was determined as described in Section 2. Values of total (extracellular plus internalised) specific binding after 30 min incubation at 37 °C for wild-type and Mut123 mutant AT_{1A} receptors were 1.5 ± 0.8 and 1.1 ± 0.6%, respectively, of the added tracer. Data represent the mean ± S.E. of three independent experiments, each performed in duplicate.

mutant and wild-type receptor (Fig. 5). Substitution of all studied lysine residues in the Mut123 mutant AT_{1A} receptor had no inhibitory effect on the initial phase of ^{125}I-Ang II endocytosis at 30 nM Ang II concentration, and after 2 min the accumulation of the internalised ligand was increased relative to that of the native AT_{1A} receptor (Fig. 5). These data suggest that ubiquitylation of the lysine residues in the serine/threonin-rich region of the carboxyl terminal tail is also not required for β-arrestin- and dynamin-independent internalisation of the AT_{1A} receptor.

4. Discussion

The present study was performed to evaluate the role of ubiquitylation of the lysine residues in the serine/threonine-rich region of the cytoplasmic tail in internalisation of the AT_{1A} receptor. Our hypothesis was that agonist-induced phosphorylation of the serine/threonine-rich region in the cytoplasmic tail of the AT_{1A} receptor could contribute to its internalisation in part by promoting the ubiquitylation of the receptor on lysine residues, as observed for certain yeast plasma membrane receptors. However, the present data demonstrate that the serine/threonine-rich region in the cytoplasmic tail of the receptor regulates its internalisation by a mechanism that is independent of receptor ubiquitylation. These findings indicate that the dynamin- and β-arrestin-dependent endocytosis of the AT_{1A} receptor via clathrin-coated vesicles, which is the predominant mechanism of AT_{1A} receptor internalisation at physiological hormone concentrations (Gáborik et al., 2001; Anborgh, Seachrist, Dale, & Ferguson, 2000; Kohout et al., 2001; Werbonat, Kleutges, Jakobs, & van Koppen, 2000), does not require ubiquitylation of the receptor. At supraphysiological Ang II concentrations, endocytosis of the AT_{1A} receptor occurs by a dynamin and β-arrestin independent mechanism (Gáborik et al., 2001; Zhang et al., 1996). Our data on the kinetics of receptor endocytosis at higher (30 nM) Ang II concentration suggest that ubiquitylation of the serine/threonin-rich region of the cytoplasmic tail is also not required for the endocytosis of the AT_{1A} receptor under these conditions.

Relatively little is known about the role of ubiquitin in the regulation of endocytosis of GPCRs in mammalian cells. Among the mammalian GPCRs, rhodopsin (Obin, Jahngen–Hodge, Nowell, & Taylor, 1996), the δ-opioid receptor (Petaja-Repo et al., 2001), chemokine receptors (Marchese & Benovic, 2001), and the β_2-adrenergic receptor (Shenoy et al., 2001) have been reported to be ubiquitylated. However, this process is involved in degradation of these receptors and does not appear to be related to receptor endocytosis. A study on the rod outer segment showed that ubiquitin attachment has a role in regulation of rhodopsin (Obin et al., 1996). This modification caused increased protein degradation but did not promote endocytosis of rhodopsin, which is normally a non-internalising receptor. Recent studies have shown that the δ-opioid receptor can undergo ubiquitylation, but in this case the polyubiquitin chain is attached before the receptor reaches the plasma membrane (Petaja-Repo et al., 2001). This process might serve to control the quality of newly synthesised receptors by targeting misfolded proteins for proteasomal degradation (Petaja-Repo et al., 2001). In the CXCR4 chemokine and β_2 adrenergic receptors, ubiquitin attachment seems to be involved in the postendocytotic processing of the receptors (Shenoy, et al., 2001; Marchese & Benovic, 2001). The CXCR4 receptor undergoes agonist-induced monoubiquitylation, but substitution of three lysine residues adjacent to a serine-rich "degradation motif" did not interfere with its endocytosis (Marchese & Benovic, 2001). Inhibition of ubiquitylation of the β_2-adrenergic receptor by replacing all of its lysine residues with arginine decreased the rate of receptor degradation but had no effect on receptor internalisation.

In mammalian cells, several tyrosine kinase and tyrosine kinase-linked receptors have been shown to undergo ubiquitylation during agonist stimulation (reviewed in Bonifacino & Weissman, 1998; Hicke, 1999). One of the best-characterised examples of the ubiquitylation of a mammalian membrane receptor is that of the growth hormone receptor. Binding of growth hormone induces dimerisation, activation, internalisation and ubiquitylation of its receptor. Studies on cells with a temperature-sensitive defect in ubiquitin conjugation, and cells expressing truncated growth hormone receptors, revealed that the ubiquitin conjugation system, but not ubiquitylation of the receptor per se, is required for agonist-induced internalisation of the growth hormone receptor (Strous, van Kerkhof, Govers, Ciechanover, & Schwartz, 1996; Sachse, van Kerkhof, Strous, & Klumperman, 2001). In contrast to endocytosis of the yeast α-factor receptor endocytosis, where ubiquitylation of the receptor itself is required for agonist-induced endocytosis (Shih et al., 2000), in the growth hormone receptor the ubiquitin conjugating enzyme complex may serve as an adaptor that links the receptor to the endocytotic apparatus. Several reports have demonstrated that the agonist-occupied EGF receptor is ubiquitinated at the plasma membrane by the RING domain-containing E3 ligase, c-Cbl, which promotes down-regulation by sorting the activated receptor to lysosomes (reviewed in Thien & Langdon, 2001). Previous studies have reported that although ubiquitylation of the EGF receptor is not involved in its internalisation, ubiquitin attachment to lysine residues of the intracellular region of the receptor promotes its down-regulation by directing the receptor to lysosomes for degradation via sorting to the internal vesicles of multivesicular bodies (Waterman & Yarden, 2001). In yeast, the ESCRT protein complex has been reported to mediate ubiquitylated cargo protein sorting into the vacuole, which is analogous to the lysosome (Katzmann, Babst, & Emr, 2001). A similar complex, which contains at least two mammalian orthologs of the yeast ESCRT-I components, might play a role in EGFR degradation in mammalian cells (Babst, Odorizzi, Estepa, & Emr, 2000).

Although our data, in agreement with studies on other mammalian GPCRs, suggest that ubiquitylation is not required for endocytosis of the AT_{1A} receptor, it remains possible that ubiquitylation has other functions in its regulation. In the receptor tyrosine kinases, down-regulation occurs by targeting receptors from the plasma membrane for subsequent sorting to the degradative pathway. The plasma membrane proteins are predominantly degraded in lysosomes, and the function of ubiquitylation is to regulate their sorting to lysosomes rather than to direct them to proteasomal degradation. The ubiquitylated receptors may interact with regulatory proteins that facilitate lysosomal sorting through the late endosome/multivesicular body compartment. Recent studies suggest that ubiquitylated plasma membrane proteins and biosynthetic cargo molecules are targeted by proteins containing specific ubiquitin recognition domains (Babst et al., 2000; Hofmann & Falquet, 2001). The recent identification of GASP as a protein that regulates sorting of the δ-opioid receptor to the degradative pathway suggests that non-covalent interactions with sorting proteins may also determine the fate of internalised GPCRs (Whistler et al., 2002). Recycling of the AT_{1A} receptor has been reported to differ from that of the β_2-adrenergic receptor, because the internalised β_2 receptor is rapidly recycled back to the cell surface whereas the AT_{1A} receptor is recycled with slower kinetics (Anborgh et al., 2000; Hunyady et al., 2002; Oakley, Laporte, Holt, Barak, & Caron, 1999). Ubiquitylation may be a mechanism for regulation of the recycling properties of the individual GPCRs. As reported for receptor tyrosine kinase receptors, ubiquitin conjugation to GPCRs may be a signal for their lysosomal degradation (Raiborg et al., 2002). In the present study, substitution of all lysine residues in the relevant carboxyl-terminal tail segment caused increased accumulation of radiolabelled Ang II at a saturating (30 nM) hormone concentration, and this was more prominent during prolonged incubation (Fig. 5). Although the reason for this phenomenon is not yet known, it is tempting to speculate that decreased targeting of the receptor to a slow recycling pathway (or to lysosomes) increases rapid recycling, causing enhanced ligand accumulation after longer incubations.

In conclusion, the present data indicate that ubiquitylation of the lysine residues in the cytoplasmic tail of the AT_{1A} receptor is not required for agonist-induced endocytosis of the hormone–receptor complex, and suggest that mammalian GPCRs are internalised by a different mechanism than that of the yeast G protein-coupled mating factor receptors. Additional studies will be required to clarify the role of ubiquitylation in the intracellular processing and long-term down-regulation of the AT_{1A} receptor and other mammalian GPCRs.

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